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Review

The BRCA1 ubiquitin ligase and homologous recombination repair

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ABSTRACT

Impairment of homologous recombination (HR), a vital process employed during repair of DNA double strand breaks and stalled DNA replication, provides a valuable opportunity for the cell to become transformed. Once transformed, the impairment turns to be a target for therapy as exemplified by the synthetic lethal strategy such as poly (ADP-ribose) polymerase (PARP) inhibitor for BRCA1/2-defective breast and ovarian cancer. Hence, improving mechanistic understanding of HR has emerged as an urgent issue to address due to the high clinical demand. Ubiquitin modification plays a central role in HR and more than a few E3 ubiquitin ligases have been implicated in the process. However, the significance of the activity of one such key E3 ligase, BRCA1, has not yet been clarified and remains as a major obstacle in the field. Here, we review recent advances in our understanding of BRCA1 function in HR and discuss possible roles of the activity.

1. Introduction

Ubiquitin modification plays crucial roles in most of, if not all, the cellular functions including proteolysis, trafficking, signal transduction, and DNA repair. The involvement of ubiquitin in DNA repair processes has been clarified by a fact that one of DNA repair genes, RAD6, was characterized as an E2 ubiquitin-conjugating enzyme [1]. Afterward Lys-63 (K63)-linked ubiquitin chains have been shown to be essential for DNA repair in yeast [2]. Two decades later it is now generally accepted that ubiquitination plays central roles in multiple DNA repair cascades and various E3 ubiquitin ligases for the ubiquitin chains have been identified with their distinctive functions. In mammalian cells homologous recombination (HR) repair is organized by at least seven E3 or E3 complexes, BRCA1/BARD1, RNF8, RNF20/40, RNF168, RAD18, HERC2, and polycomb-repressive complex 1 [3–15]. The significances of the activities have been shown for some of the E3 ligases. In contrast, the significance of the E3 ligase activity of BRCA1 (breast and ovarian cancer susceptibility protein 1) is still obscure, despite it was the first mammalian E3 enzyme shown to function at sites of DNA damages [16,17]. BRCA1 forms a RING heterodimer with BARD1 (BRCA1-associated RING domain 1), an interaction necessary for BRCA1 protein stability, nuclear localization, and the E3 ligase activity [16,18–20]. Both BRCA1 and BARD1 are absolutely required for HR and other critical cellular events in response to DNA damage, and loss of either protein results in susceptibility to breast cancer. However, how the E3 ligase activity contributes to these events, or even whether the activity is indeed inevitable, would be controversial. In this review, we have summarized current knowledge concerning BRCA1 functions in HR, and attempted to interpret the role of BRCA1's E3 ligase activity in the process.

2. BRCA1 functions in HR

The essential role of BRCA1 in response to DNA damage has first been shown by mouse embryonic stem (ES) cells or fibroblasts (MEFs) carrying a targeted deletion of exon 11 of the *BRCA1* gene. Exon 11 is the largest exon required for the interaction with RAD51, a critical effecter for HR [21], and the cells lacking the exon were defective for G2/M checkpoint [22] and DNA double-strand breaks (DSBs) repair by HR [23]. The *BRCA1* gene encodes a protein of 1863 amino acids [24]. The protein consists of a RING finger domain in its N-terminal region and tandem BRCT domains in its C-terminal region in addition to a region encoded by the exon11 in the middle. The N-terminal RING finger domain interacts with BARD1, which also contains an N-terminal RING domain and C-terminal tandem BRCT domains [25,26]. The BRCT repeat of BRCA1 is a phospho-protein binding module and interacts with phosphorylated Abraxas, BACH1 (also called FANCJ or BRIP1) and CtIP in mutually exclusive manner, and thereby contributes to the formation of three distinct complexes, called BRCA1-A, B, and

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C [27–31]. BRCA1 also constitutes a complex with BRCA2 through an interaction bridged by PALB2 [32,33]. Thus BRCA1 forms several key protein complexes that distinctively and cooperatively function in response to DNA damage. The BRCA1–BARD1 RING heterodimer exists in all the BRCA1 complexes as a core component. Key functions of each complex in response to DNA damage have gradually been revealed.

2.1. DSB end-resection by BRCA1-CtIP

The site of DNA damage is recognized by damage sensor complex comprising Mre11, RAD50 and NBS1 (MRN) in the initial steps of HR [34]. Mre11 has endonuclease and exonuclease activities [35,36] (Fig. 1). MRN promotes the recruitment of ATM to DSBs that is essential for phosphorylation of histone H2AX and subsequent phosphorylation of MDC1 that allows RNF8 cascade [34,37,38]. In addition MRN recruits BRCA1-CTIP through direct binding to phosphorylated CtIP and forms BRCA1-C complex [39]. BRCA1-CtIP, in conjunction with the MRN complex, mediates extensive DSB end resection that generates single strand DNA (ssDNA) overhangs to support subsequent HR-mediated repair of DSB [40,41].

On the other hand CtIP is capable of generating limited DSB end resection without BRCA1 to promote altered non-homologous end joining (alt-NHEJ), an error-prone repair in G1 phase of cell cycle mediated by the annealing of microhomology regions [42]. The switch from alt-NHEJ to HR is mediate by phosphorylation of Ser 327 of CtIP in S-phase that promotes BRCA1 interaction (Fig. 1). The DSB end resection promoted by CtIP is inhibited by 53BP1, and BRCA1 overwhelm 53BP1 to execute the resection [43]. This inhibition is important for protection of alt-NHEJ in G1-phase when CtIP does not interact with BRCA1, to support Ku-mediated NHEJ [44]. In addition 53BP1 blocks HR and sustain the growth arrest induced by BRCA1 depletion [43,45]. Interestingly loss of 53BP1 partially rescues the BRCA1 deficiency and is associated with breast cancer development [45]. Together, one major function of BRCA1 in the BRCA1-C complex is suppression of 53BP1 and prolongation of the CtIP activity for DSB end resection to generate ssDNA length long enough for HR.

2.2. Recruitment of BRCA1 by K63-linked ubiquitin conjugates

K63-linked polyubiquitin chains assembled on PCNA plays critical roles in postreplication DNA repair. In addition their

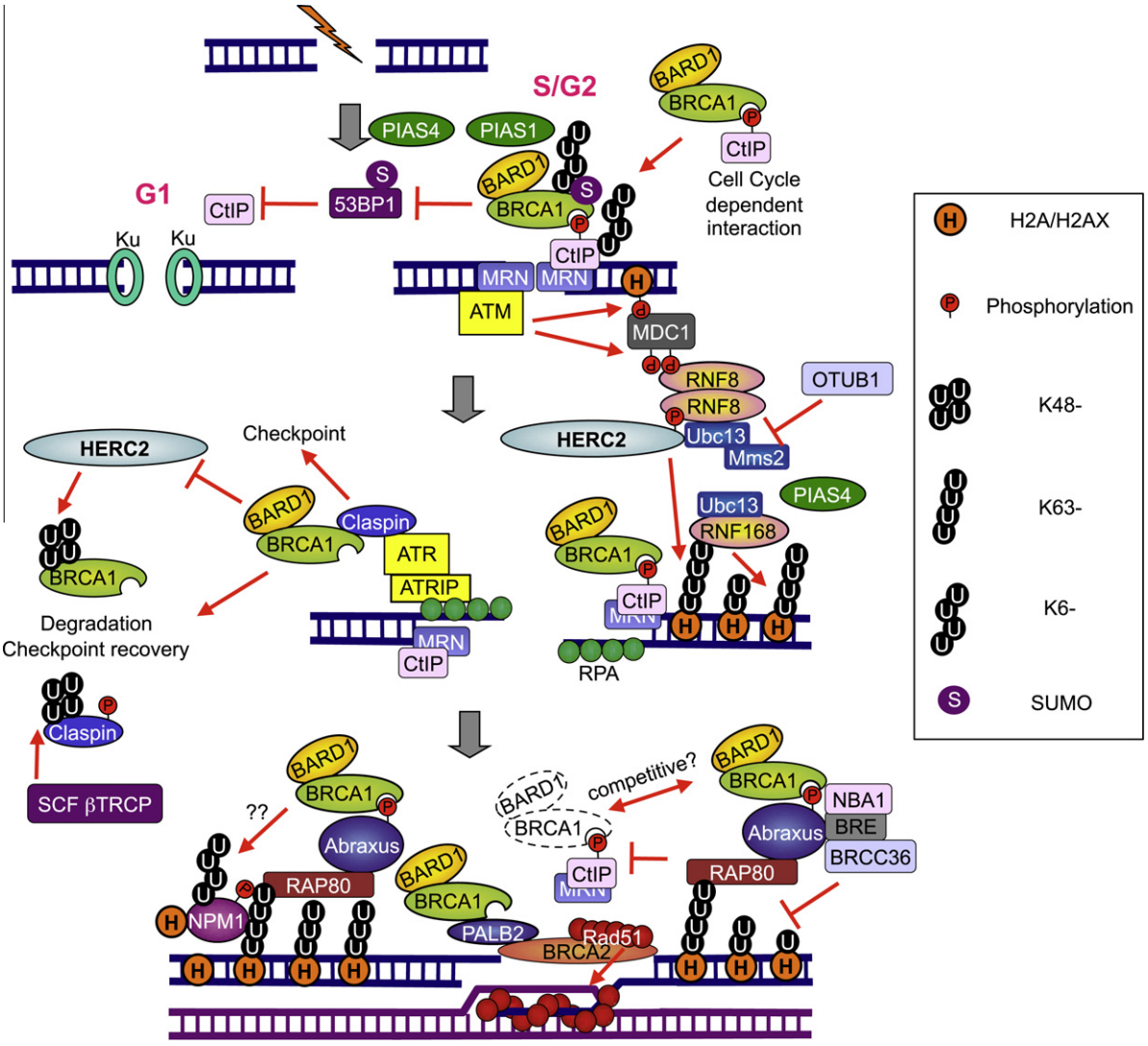

Fig. 1. Homologous recombination repair pathway for DSBs carried out by ubiquitin modification focused on the functions of BRCA1-CtIP (BRCA1-C) and BRCA1-RAP80 (BRCA1-A) complexes. Various E3 ubiquitin ligases, DUB enzymes, and E3 SUMO ligases are involved in the process. Details are described throughout the text.

involvement in HR has been elucidated by a discovery of a function of RAP80 that recruits BRCA1 to the sites of DSBs [29,46,47] (Fig. 1). RAP80 constitutes BRCA1-A complex (RAP80-Abraxas-BRCA1) and directs the complex to the K63-linked ubiquitin chains through its tandem UIM (ubiquitin-interacting motif) domains (Fig. 1). Subsequently RNF8 was discovered to be a RING type E3 ligase that is recruited to DSBs in a manner dependent on ATM phosphorylation of MDC1 and generates K63-linked ubiquitin chains required for the downstream HR process [4–7]. The ubiquitin products mediated by RNF8 were not sufficient for exertion of HR, and RNF168, another RING type E3 ligase, was later found to enhance the K63-linked polyubiquitination [8,9]. RNF168 contains MIU (motif interacting with ubiquitin) domain and directly interacts with K63-linked ubiquitin conjugates initially generated by RNF8.

Components in the BRCA1-A complex including Abraxas, BRE/BRCC45, BRCC36, and NBA1/MERIT40 were also shown to directly interact with ubiquitin conjugates *in vitro* through MPN, Uev, MPN+, and VWA domains, respectively [48]. NBA1/MERIT40 integrates the complex through maintaining the stability of BRE/BRCC45 and is required for BRCC36-mediated deubiquitinating enzyme (DUB) activity in the complex [49,50]. In addition to the above ubiquitin-interacting proteins, nucleophosmin (NPM1, also called B23), a candidate substrate for BRCA1 E3 activity [51], is recruited to DSBs in a manner dependent on the RNF8/RNF168-mediated ubiquitin conjugates [52] (Fig. 1). Significantly NPM1 interacts with K63-linked ubiquitin polymer *in vitro* with high affinity. The affinity relies on phosphorylation of Thr199, an acidic tract, and an adjacent UIM-like domain within NPM1 [52]. All the components recruited by the ubiquitin conjugates are likely essential for efficient HR. Specific tasks for individual proteins in the process have progressively been elucidated.

Production of the K63-linked polyubiquitin chains by RNF8 and RNF168 are regulated by complex mechanisms through accessibility of their vital E2 enzyme, UBC13. HERC2, a HECT type E3 ligase and a DUB OTUB1 have been involved in the mechanisms (Fig. 1). HERC2, an enormously large 4834 amino acid protein comprising three RCC1-like domains and a C-terminal HECT domain, interacts with the FHA domain of RNF8 in a phosphorylation-dependent manner and facilitates assembly of the RNF8-UBC13 complex [11]. It also maintains the level of RNF168. In contrast to HERC2, OTUB1 binds to and inhibits UBC13 and thereby suppresses RNF168-dependent polyubiquitination [53]. Interestingly neither HERC2 nor OTUB1 requires its enzymatic activity, i.e., E3 ligase activity or DUB activity, respectively, to regulate RNF8 and RNF168. This may suggest that they further contribute to DSB repair process on different aspects by regulating ubiquitination status with the specific activities. One such function for HERC2 could be its action toward BRCA1 as described later.

Although precise mechanisms for the ubiquitin-dependent recruitment of RAP80-Abraxas-BRCA1 (BRCA1-A) complex has been elucidated as described, the role of BRCA1 in this pathway is not clear. In this regards, an unexpected function of this complex, where it compete with the other complexes for BRCA1, was recently discovered (Fig. 1). Whereas RAP80 is required for ionizing radiation-induced foci (IRIF) formation of BRCA1 at late stage after IR, it is dispensable for the IRIF formation at early stage, until 1 h after IR [54]. The early BRCA1 recruitment to DSBs is likely mediated by CtIP and BACH1. Interestingly RAP80 depletion led to significantly higher rate of DSB end processing as detected by IRIF formation of ssDNA binding protein RPA or that of BrdU in non-denaturing condition [54,55]. It is accompanied by enrichment of CtIP, BACH1 and RAD51 at DSBs, and leads to an increased frequency of HR in reporter cells [54,55]. Importantly the excess HR activity renders cells to develop gross chromosomal rearrangements [54]. This suggests that appropriate tuning of BRCA1 activity for DSB end resection and HR is critical for genome integrity maintenance. BRCA1-A complex perform this task by suppressing BRCA1-C complex.

2.3. Recruitment of RAD51 recombinase by BRCA1

DNA strand invasion into homologous sequences of the sister chromatid, the main event for the reaction of HR repair is carried out by recombinase RAD51 [56] (Fig. 1). The ssDNA binding protein RPA first interacts with and occupies the 3′ single strand overhangs generated by the BRCA1-CtIP. RAD51 next replaces RPA and constitutes ssDNA-RAD51 filament under guidance of BRCA2, which is capable of holding several RAD51 molecules through 8 BRC repeats [57,58]. The RAD51 filament then searches for the homologous region. BRCA1 is essential for the retention of BRCA2 and RAD51 at the DSB site [59]. This is one of the principal roles of BRCA1 in the HR process in addition to the DSB end resection.

The recruitment of BRCA2 by BRCA1 is mediated by PALB2, which acts as a linker between the two proteins [32,33] (Fig. 1). However, how the BRCA1–PALB2–BRCA2 complex is recruited to the DSB sites is obscure. Because PALB2 binds to BRCA1 outside of its BRCT domains, it is possible that one of three BRCA1 complexes, i.e., A, B and C, may contain the BRCA2-RAD51. However, depletion of neither RAP80 nor BACH1 abolishes the IR-induced RAD51 foci formation [54,60]. In addition neither BACH1 nor RAD50 coimmunoprecipitated BRCA2, and reciprocally BRCA2 did not coimmunoprecipitate either protein while it did RAD51 [59]. These results suggest that the retention of BRCA1–PALB2–BRCA2 at the site of DNA damage is independent of other three BRCA1 complexes. Alternatively PALB2–BRCA2 may replace other BRCA1-interacting proteins after BRCA1 is recruited to the DSB sites. Mechanism organizing the order of accumulation of the four BRCA1 complexes to recruit BRCA2-RAD51 is a critical issue to be elucidated.

3. E3 ligase activity of BRCA1 in HR

One remarkable feature for BRCA1 is its E3 ligase activity that is acquired by constitution of a RING heterodimer with BARD1, a core complex residing in most of BRCA1 complexes [16,59]. Because all four BRCA1 complexes critical for HR contain this core complex, the activity is likely important for the process. However, there are some hardships to obtain direct evidence for the significance of the activity. Nonetheless several indirect evidences suggest its requirement.

3.1. Is BRCA1 E3 activity required for HR?

The potential importance of the E3 ligase activity of BRCA1 in cellular pathways was supported by the fact that many missense mutations within RING finger domain of BRCA1, which causes familial breast cancer, abolished the E3 activity [16,18,61]. These mutants are unable to restore gamma-radiation sensitivity, G2/M checkpoint activity and HR in BRCA1 defective cells [61,62]. However, most of the mutations that eliminate the activity also abolish the interaction with BARD1. Because the *in vivo* stabilities and nuclear localization of BRCA1 and BARD1 are interdependent [16,19,20,63], the phenotype observed in these mutations are attributed to the total BRCA1 deficiency. Indeed, mouse models with mammary epithelial cells targeted ablation of BRCA1, BARD1, or both developed breast carcinomas that are indistinguishable among the genotypes with respect to their frequency, latency, basal-like histopathology, and cytogenetic features [64].

In order to analyse the genuine significance of the E3 activity, mutations that only abolish the activity but does not affect other functions including the protein stability or BARD1 binding is ideal.

Investigation of multiple mutants of BRCA1 from patients that disrupt the interaction of E2 enzymes without perturbing the BRCA1–BARD1 complex revealed that the E3 ligase activity strongly correlates with breast cancer susceptibility [65]. However, to our surprise, genetically engineered mouse ES cells expressing BRCA1 with substitution of alanine for isoleucine at position 26 (I26A), a popular mutation to disrupt the E2 binding without perturbing BARD1 binding [18], did not exhibit HR failure [66]. The ES cells with BRCA1 I26A were resistant to genotoxic stress, were capable of accumulating RAD51 at DSBs, and mediated HR repair at the same level as cells with wild type BRCA1. Nonetheless a few questions have been evoked before conclude that E3 activity of BRCA1 is dispensable for HR.

First, DNA repair capacity of ES or stem cells is different from that of differentiated cells [67,68]. To maintain the genetic stability in daughter cells, ES cells must have robust mechanisms to protect their DNA from damages. Basically ES cells are proficient in HR as evidenced by their use in gene targeting experiments. They grow quickly with very short G1 and G2 phases and express much higher level of RAD51 than MEFs do [68]. It has been reported that overexpression of RAD51 in BRCA1 deficient DT40 cells rescues defects in proliferation, DNA damage survival, and HR [69]. Together, the demand for BRCA1 in the ES cells could be less than that in differentiated cells. Indeed the expression level of BRCA1 I26A in the ES cells was considerably low while the cells were intact for HR [66].

Second possibility is that the E3 ligase activity of BRCA1 could be required for HR process in response to some specific type of DNA damage. The DNA damage utilized in the previous report was that introduced by either IR, mitomycin C (MMC) or the restriction enzyme I-SceI [66]. These agents induce either DNA cross-linking or DSB. However, other types of DNA damage that employs HR in the process, such as that induced by PARP inhibitor may requires BRCA1 E3 activity.

Third, the ES cells expressing inactive BRCA1 E3 ligase showed elevated level of damage-induced, but not spontaneous, chromosomal abnormalities. The mutant cells showed MMC-induced chromosomal aberration at higher level than wild type BRCA1 [66]. This suggests that the DNA repair system in the cells is not totally intact although the luck of function could not be detected by other procedures. It will be important to determine whether the observed genetic instability is sufficient to cause breast cancer in the mice.

Lastly the mutations in RING finger of BRCA1 that affect the E3 ligase activity were also investigated in HeLa cells for HR competence. Interestingly some mutations that disrupt the interaction of E2 enzymes without perturbing the BARD1 binding, including T18M, T37R and H41R [65] (Nishikawa and Ohta, unpublished data), remarkably inhibited HR [62]. Collectively the significance of E3 ligase activity of BRCA1 in HR needs to be further investigated in different settings.

3.2. Ubiquitin conjugates at DSBs mediated by BRCA1

Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) and is capable of forming seven distinct types of chains [70]. In addition linkage through C-terminal glycine of a ubiquitin and N-terminal methionine of another ubiquitin creates linear ubiquitin chains [71]. While K11 and K48 chains signal for proteolysis by the proteasome, K63-linked and linear chains as well as mono-ubiquitination regulates the assembly of protein complex. However, the ubiquitin chains catalyzed by BRCA1 were shown to be K6-linked *in vitro* using UbcH5 as E2 enzyme, or *in vivo* using cells overexpressing BRCA1, BARD1 and mutant ubiquitins [51,72,73]. The K6-linked ubiquitin chains are not recognized by proteasome and likely signals other than proteolysis [72]. Adversely K6-linked chains could stabilize substrates because BRCA1 mutants that lack E3 ligase activity and therefore fails to be auto-ubiquitinated are relatively unstable [66]. The most conceivable function of K6-linked chains at present is to assemble protein complexes that are critical for HR process, similar to K63-linked chains.

Earlier reports showed that ubiquitin structure accumulated at DSBs after IR was BRCA1 dependent and was inhibited by BRCA1 depletion by siRNA or by the expression of ubiquitin with K6R mutation [17,74]. The ubiquitin at DSBs were detected by FK2 antibody that exclusively recognizes conjugated ubiquitin. Later studies demonstrated that the FK2-IRIF were detected even in BRCA1 depleted cells [6]. The primary E3s that is required for the FK2-IRIF is now known to be RNF8 and RNF168 as described above. However, because knockdown of BRCA1 reduced the FK2-IRIF [6], it is likely that BRCA1-mediated ubiquitination occurs at the sites of DNA damage in addition to RNF8/168-mediated ubiquitin conjugates. The K6-, in addition to K63-linked chains, are capable of interacting with RAP80 and therefore may contributes to recruit BRCA1-A complex [47]. Together one may consider that RAP80 (BRCA1-A) complex acts as positive feedback loop for accumulation of the ubiquitin conjugates. However, interestingly, inhibition of RAP80, in addition to BRCC36, a DUB subunit in BRCA1-A complex, remarkably enhances the accumulation of conjugated ubiquitin at DSBs [75]. This suggests that the DUB activity mediated by BRCC36 carries greater weight than the ubiquitination mediated by BRCA1 in the RAP80 complex in response to DNA damage, and could be consistent with the role of BRCA1-A complex as a negative regulator for BRCA1-CtIP-mediated end resection as described above (Fig. 1). If this is the case, BRCA1-CtIP and/or BRCA1–BACH1 complexes, instead of BRCA1-RAP80 complex would be responsible for the BRCA1-induced enhanced FK2-IRIF in normal setting.

BRCA1 is capable of creating K63- or K48-linked chains *in vitro* in combination with distinct E2s, UBC13/MMS2 or UBE2K, respectively, in addition to K6-linked ubiquitin chains [76]. Mono-ubiquitination by UBE2W on substrates is required as a first step in the reaction that is subsequently recognized by the second E2s, UBC13/MMS2 or Ube2K, whereas UBCH5-mediated K6-linked ubiquitin polymers do not require the initial E2. UBCH5c is recruited to the sites of DNA damage after IR and interacts with BRCA1 in UBC13 dependent manner [74,77]. Whether UBE2w and UBE2K also acts in response to DNA damage remains to be determined.

3.3. Candidate substrates in HR

To uncover the significance of the E3 ligase activity of BRCA1 in HR, the identification of its bona fide substrate(s) in the process is probably the most important step. However, several hardships have been keeping researchers including us away from the direct evidences to prove it since the activity was discovered 10 years ago [16]. Such evidences may include: (a) BRCA1 dependent ubiquitination of endogenous substrate(s) at DSBs under physiological condition. (b) Direct *in vitro* ubiquitination of substrate(s) by BRCA1–BARD1 in pure recombinant system. (c) Demonstration of physiological significance of the substrate ubiquitination in BRCA1-related functions.

Difficulties to demonstrate each evidence may include: (a) Outcome of the assay heavily rely on the efficacy of the antibody used for immunoprecipitation which may require dinature condition; preparation of soluble chromatin fraction is required before immunoprecipitation; prevention of contaminated DUB activity is inevitable; cells with or without BRCA1 (ideally BRCA1 E3 activity), possessing otherwise identical genetic backgrounds are essential. (b) Purification of full-length dimer, or shorter fragments that contain the substrate recognition site, are required; Bona fide E2 is not totally clear; If UbcH5 is used, low-grade ubiquitination could be observed for many different non-specific substrates

in vitro. (c) One possible way to show the physiological significance is determination of the phenotype of a ubiquitin-insensitive mutant of the substrate. However, UbcH5c self-assembles E2-ubiquitin oligomer through non-covalent interaction with ubiquitin in addition to a covalent thiolester with the C-terminus of ubiquitin that allows multiple mono- or poly-ubiquitination at many lysine residues on a single protein *in vitro* [78,79]. Therefore identification of the specific ubiquitin-binding site(s) is almost impossible; mutation that inhibits E3-substrate interaction could be valuable.

So far there are no substrates that perfectly satisfy the criteria. Nonetheless, two potential substrates, CtIP and NPM1 that are involved in HR have been demonstrated. Phosphorylated CtIP interacts with BRCT domains of BRCA1 and plays critical role in DSB end resection in HR as described above.

CtIP can be ubiquitinated by BRCA1–BARD1 *in vitro* and *in vivo* [80]. The ubiquitinated CtIP in the chromatin extraction was clearly observed after IR in BRCA1-deficient HCC1937 cells only when enzymatically active BRCA1 was exogenously expressed. The ubiquitination depends on Ser 327 residue of CtIP, the phosphorylation site essential for interaction with BRCA1 BRCT domain. Importantly only wild-type BRCA1, but not the I26A E3 ligase mutant of BRCA1, restored CtIP IRIF formation. The results suggests that BRCA1-mediated ubiquitination of CtIP is required for its retention on chromatin (Fig. 1). Ubiquitination of BACH1 and RAP80, the other two phosphorylation-dependent binding partners for BRCA1, have not yet been reported.

Another candidate substrate, NPM1, was initially identified as a substrate for BRCA1 by two different screen methods using mass-spectrometry [51]. One is a screen of BRCA1 I26A immunocomplex from a cell lysate. The other is a screen of FLAG-ubiquitin immunocomplex precipitated from a supernatant of ubiquitin ligation reaction containing FLAG-ubiquitin and BRCA1 immunoprecipitation. Recently it was shown that NPM1 phosphorylated at Thr199 is recruited to DSBs by RNF8/RNF168-mediated ubiquitin conjugates [52] (Fig. 1). IR-induced direct or close interaction between BARD1 and NPM1 at DSBs was demonstrated as Venus fluorescent foci, which is comprised of BARD1 and NPM1 fused to truncated N- and C-terminus of Venus protein, respectively. Importantly replacement of endogenous NPM1 with its non-phosphorylable T199A mutant prolongs the persistence of IR-induced RAD51 foci accompanied by unrepaired DNA damage.

NPM1 is an abundant nucleolar phosphoprotein that participates in multiple cellular processes including ribosome biogenesis and transport, possesses anti-apoptotic activity, regulates centrosome duplication, and plays crucial roles in ARF-MDM2-p53 pathways [81]. Structural and biochemical analyses demonstrated that NPM1 is a decameric histone chaperone that interacts with core histones [82–84]. It is clinically important in haematopoietic tumours because chromosomal translocations resulting in chimeric NPM1-kinase fusion proteins cause anaplastic lymphomas [85,86], and mutations generating a C-terminal extension are frequently found in acute myeloid leukemia [87]. NPM1 is efficient substrate for BRCA1 either *in vitro* [52,88] or *in vivo* using exogenously expressed proteins [51]. However, BRCA1 dependent ubiquitination of endogenous NPM1 in response to DNA damage remains to be demonstrated.

Other previously reported candidate substrates for BRCA1 include histones, FANCD2, the largest subunit of RNA polymerase II (RPB1), γ-tubulin, estrogen receptor α, the common subunit of RNA polymerases RPB8, progesterone receptor-A, and the general transcription factor TFIIE [89–96]. Of these closely relevant to HR were histones and FANCD2. Mono-ubiquitination of FANCD2 is a key step in DNA cross-link repair mediated by Fanconi anemia complex. However, FANCL has been later discovered as a bona fide E3 for FANCD2 and BRCA1 was ultimately excluded from the candidate E3 responsible for the reaction [97–99].

Histone H2A and H2AX were used as model substrates for BRCA1 E3 ligase activity *in vitro* in earlier reports [61,100]. They were mono-ubiquitinated in the reactions with E2 Ubc4 or UbcH5c. Later histones H2B, H3, and H4, in addition to H2A and H2AX were shown to be mono-ubiquitinated *in vitro* with mixture containing BRCA1–BARD1 and UbcH5a, whereas histone H1 was not [101]. This may suggest some specificity, however, UbcH5 type E2s are promiscuous *in vitro* and low level of ubiquitination including mono-ubiquitination can be observed on many different substrates as described above [78]. *In vivo* evidence for BRCA1-mediated ubiquitination of these histones has not yet been demonstrated. Instead γ-H2AX can be mono-ubiquitinated after IR in BRCA1−/− DT40 cells although the level of ubiquitination is reduced [77]. In addition bona fide E3s for mono- or polyubiquitination of H2A, H2AX and H2B in HR process have been identified. K63-linked polyubiquitination of H2A and H2AX essential for recruitment of BRCA1-RAP80 is primarily mediated by the collaboration of RNF8 and RNF168 [4–7,9102]. In addition mono-ubiquitination of H2A and H2B is dramatically reduced in RNF8-deficient MEFs, which abolishes BRCA1 accumulation at DSBs [103]. The ubiquitination of H2B in response to DNA damage may not be direct effect of RNF8, because RNF20 and RNF40 were found to be the E3 responsible for the reaction [12,13]. The RNF20/40-mediated H2B ubiquitination at lysine120 renders DNA relaxation allowing subsequent DSB end resection and recruitment of BRCA1 and RAD51. Another E3 that has recently been demonstrated as an E3 responsible for γ-H2AX ubiquitination is BMI1, a subunit of polycomb repressive complex 1 [14,15]. BMI1 is recruited to DSBs and is requited for DNA damage-induced ubiquitination of H2A at lysine 119 as well as for recruitment of RAP80 and BRCA1.

Thus major role of ubiquitination of H2A and H2B is likely to create initial ubiquitin conjugates scaffold and to generate chromatin relaxation that are required for recruitment of downstream repair proteins including BRCA1. If BRCA1 also ubiquitinates the same substrates after the initial ubiquitination, the specific role of the activity would be obscure. It is not conceivable that BRCA1 ubiquitinates the histones to extend the relaxed chromatin in the frank regions of DSBs because the role of BRCA1–RAP80 complex that is recruited to DSBs at late phase, is to suppress, rather than create, the end resection [54,55]. Nonetheless, it is possible that additional relaxation of chromatin could be required for other processes including the strand invasion or polymerization of DNA.

4. Regulation of BRCA1 E3 ligase in HR

The activities of many kinases are up or downregulated in response to DNA damage by covalent modification such as phosphorylation at a critical residue that renders conformational alteration as exemplified by ATM and Chk2 [104,105]. On the other hand, such a regulation of E3 ligases in HR process has not been common. However, some important modifications or protein interactions that regulate the E3 ligase activity of BRCA1 has been demonstrated.

4.1. Sumoylation

The importance of SUMO modification in HR has been revealed by the requirement of PIAS1 and PIAS4, the E3 ligases for SUMO, in the process [106,107] (Fig. 1). PIAS1 and PIAS4 are recruited to DSBs in a SAP domain dependent manner, and generate accumulation of SUMO1, 2, and 3 at the sites. Depletion of PIAS1 or PIAS4 reduced the proportion of cells displaying BRCA1 accumulation and decreased BRCA1 staining intensity at DSBs. PIAS1 or PIAS4 depletion also dramatically impaired ubiquitin-conjugate accumulation detected by FK2 antibody whereas RNF8 accumulation appeared

normal. Interestingly, however, recruitment of RNF168 was impaired only in PIAS4 depleted cells but not in PIAS1 depleted cells. Because 53BP1 recruitment does not require BRCA1- or PIAS1-mediated intended ubiquitin conjugates but requires PIAS4, it may rely on mono-ubiquitination of histones mediated by RNF168. In this regards, the amount of focal ubiquitination may determine the pathway to repair DSBs, NHEJ or HR. Importantly SUMO modification of BRCA1 significantly increases the E3 ligase activity of BRCA1–BARD1 heterodimer *in vitro*, and mutations at the sumoylation sites of BRCA1, K199R and E121R, dramatically reduced the conjugated ubiquitin foci formation [107].

4.2. Phosphorylation

BRCA1 is phosphorylated by several kinases involved in either cell cycle regulation or DNA damage response, including CDK2, ATM, ATR, Chk2, and CK2 [108–112]. BARD1 is also phosphorylated by CDK2, or at ATM/ATR consensus sites in response to DNA damage [113,114]. While these kinases have been shown to regulate the distinct functions of BRCA1, the direct regulation of BRCA1 E3 ligase activity by the kinases through phosphorylation of either BRCA1 or BARD1 has not demonstrated. Overexpression of CDK2 causes BARD1 phosphorylation at multiple sites in its N-terminal regions and inhibits the E3 ligase activity of BRCA1–BARD1 *in vivo* [113]. However, unphosphorylatabole mutant of BARD1 can still be inhibited by CDK2.

4.3. Deubiquitination

In general E3 ligases interacts with DUBs and coordinately regulates ubiquitination levels in cellular pathways. The DUBs shown to act in HR are BRCC36 and OTUB1. Whereas the role of DUB activity of OTUB1 is not apparent at present as described above, the DUB activity of BRCC36 likely plays significant role in BRCA1-A complex, because inhibition of BRCC36 results in enhanced conjugated ubiquitin accumulation at DSBs [75]. Whether the activity is toward ubiquitin conjugate mediated by BRCA1 or that mediated by RNF8/RNF168 is not clear. However, because inhibition of RAP80 that prohibits recruitment of both BRCC36 and BRCA1 also results in the enhanced conjugated ubiquitin accumulation [75], and because BRCC36 is K63-specific DUB [115], it is likely that BRCC36 regulates RNF8/RNF168-mediated ubiquitination. BRCC36 may also have a role to deubiquitinate K63-linked chains mediated by BRCA1-UBC13 in the BRCA1-A complex to lead K6-linked ubiquitin chains predominant.

Another DUB that possibly cooperates with BRCA1 in HR is BAP1 (BRCA1 associated protein 1), a nuclear-residing ubiquitin C-terminal hydrolase (UCH). BAP1 had been originally identified as a protein that interacted with the RING finger domain of BRCA1, before the E3 ligase activity of BRCA1 has been discovered [116]. BAP1 also directly interacts with BARD1. Interestingly, BAP1 inhibits the E3 ligase activity by interfering the RING heterodimer formation by BRCA1 and BARD1, in addition to that it deubiquitinates the ubiquitin conjugates catalyzed by BRCA1–BARD1 [88]. There is no clear evidence for BAP1 involvement in HR. However, BAP1 has been identified by proteomic analysis as a protein phosphorylated in response to DNA damage on consensus sites recognized by ATM and ATR [117]. Recently mutations of BAP1, including that perturb BARD1 interaction, were identified to be significantly correlate to metastatic uveal melanomas of the eye [118].

4.4. Inhibiting E2

E2 enzymes that interact with E3s do not always activate the ubiquitin ligation reactions. Indeed UbcH7, which interacts with RING finger domain of BRCA1 with high affinity, do not exhibit any activity with BRCA1–BARD1 *in vitro* [18] (Nishikawa and Ohta, unpublished data). Interestingly, UBXN1, an E2 that inhibits BRCA1–BARD1 mediated ubiquitin ligation, was identified. UBXN1 contains a ubiquitin-associated (UBA) motif in its N-terminus that binds K6-linked polyubiquitin chains conjugated to BRCA1 by autoubiquitination while the C-terminus of UBXN1 binds the BRCA1/BARD1 heterodimer in a ubiquitin-independent fashion [119]. Physiological relevance of the inhibition by UBXN1 remains to be determined.

4.5. Protein degradation

Unlike some E3 ubiquitin ligases, such as MDM2, that determine their fate by autoubiquitination and self-inflicted degradation as a negative feed back mechanism, the autoubiquitination of BRCA1 does not cause degradation [120]. Because the stability of BRCA1 and BARD1 are interdependent while they acquire the E3 ligase activity and are autoubiquitinated, the ubiquitination may stabilize both proteins [16,19,100]. On another front, BRCA1 is unstable protein when BARD1 expression is inhibited. BRCA1 possesses a degron domain in its C-terminal flanking region of the RING finger that leads BRCA1 to proteasome-mediated degradation [121]. Importantly a HECT type E3 ligase that interacts with the degron domain of BRCA1 and ubiquitinates and degrades BARD1-uncoupled BRCA1 was recently discovered [122]: that was HERC2, a protein essential for RNF8-mediated HR process [11].

In physiological condition steady-state level of BRCA1 protein is elevated throughout the mitosis. The turnover of BRCA1 and proteasome-sensitive ubiquitin conjugates of BRCA1 are up-regulated during G1 and S phases, and BARD1 inhibits the modification [123]. HERC2 interacts with BRCA1 mainly during S-phase and interestingly, the binding partner for BRCA1 was switched from HERC2 to BARD1 as cells enter mitosis accompanied by an increase in the BRCA1 steady state level [122]. In addition BARD1 protect BRCA1 from HERC2-mediated ubiquitination (Fig. 1). Interestingly BRCA1 destabilization attributed to BARD1 depletion by siRNA causes G2 checkpoint failure and additional HERC depletion restores the checkpoint accompanied by BRCA1 stabilization [122]. This may suggest that HERC2 regulates checkpoint recovery or cell cycle control by targeting BRCA1 for degradation. In response to IR treatment HERC2 is recruited to DSBs and plays critical role in HR as described above. In this complex BRCA1 is likely protected from HERC2 by co-existing BARD1 and is capable of supporting Claspin-mediated DNA replication checkpoint or G2 checkpoint activation [124]. Upon completion of DNA repair phosphorylation of Claspin by Plk1 triggers its $SCF^{\beta TrCP}$-mediated degradation: a central mechanism for the recovery from the checkpoint to cell cycle [125]. What triggers BARD1 dissociation from BRCA1 or how BARD1 is inhibited in physiological condition is remained to be determined. In this regard, it is intriguing that BARD1, but not BRCA1, is a substrate for anaphase promoting complex APC/C for ubiquitination and degradation [126].

5. Conclusions

Impairment of many factors involved in HR each contributes to susceptibility to breast and ovarian cancer. They also have a critical impact for chemo or radiosensitivity. As BRCA1 and BRCA2 contribute to HR at different impact, the phenotypes caused by deficiency of these genes are different. Whereas most of BRCA1 deleterious mutations cause basal-like breast cancer, the phenotype of BRCA2 mutations is Luminal A breast cancer. Likewise, as BRCA1 total deletion and BRCA1 lacking E3 ligase activity may differently affect HR competence, the phenotype and chemosensitivity attributed to these defects would also be distinctive. Mutations that eliminate

the E3 ligase activity of BRCA1 and multiple factors that affect the activity may determine the chemosensitivity to certain agents in such phenotypes. Coordination of basic and clinical research further activates the development of this field.

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